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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A CALMODULIN FROM AVOCADO FRUIT TISSUE

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Abstract—The calcium-binding proteins from avocado fruit mesocarp were investigated and a protein identifiable as a calmodulin was purified to homogeneity by *int. al.* calcium induced hydrophobic interaction chromatography. The protein has a M_r of 18 900 and exhibits a calcium dependent shift in electrophoretic mobility. Calcium induced changes in UV, fluorescence, and circular dichroism spectra were also observed. A helical content of 17% for the calcium depleted state was calculated which increased to 24% in the presence of saturating concentrations of calcium. The amino acid composition of the protein was closely related to that of the higher plant calmodulins so far analysed. Isoelectric focussing resolved the protein into a doublet with pI values of 3.71 and 3.72. The protein stimulated calmodulin-deficient NAD kinase to the same extent as calmodulin from wheatgerm, but exhibited a very low level of activity as an activator of cyclic monophosphate phosphodiesterase. The similarity and dissimilarity of the avocado protein to other calmodulins are described and discussed.

INTRODUCTION

The ripening of fruit tissue is a co-ordinated process of biochemical differentiation. Calcium is strongly implicated in the regulation of the cellular processes in ripening and senescence [1]. Probable sites for Ca^{2+} action are changes in transmembrane flux with consequent changes in cytosolic free Ca^{2+} concentrations, and changes in membrane and protein binding of Ca^{2+}. The interaction of Ca^{2+} with various proteins with regulatory functions has recently come under scrutiny [2]. A study of such interactions will help to elucidate the sequence of events leading from the initial stimulus to ripening and the final stages of senescence.

Calmodulin is a protein with all the desired characteristics for modulating calcium action [2]. It has been purified and characterized from a number of sources [2–6]. The protein binds calcium with high affinity and interacts with various enzymes, regulating their activity [2, 7, 8]. Plant enzymes known to undergo calcium- and calmodulin-regulated activation or deactivation include the following: phospholipase [8], NAD kinase [7], phosphofructokinase [9], quinate-NAD oxidoreductase [10], protein kinases [11], ATPase [12] and nitrate reductase [13].

In the avocado, various studies have shown that calcium can influence the ripening process, as well as limit the extent of physiological disorders such as browning [1]. The distribution of cellular calcium and its interaction with calcium modulating proteins seem to be one of the important factors in ripening [1, 2, 14]. It has been suggested that an important part of the senescent process is a breakdown in Ca^{2+} regulation, with an increase in intracellular Ca^{2+} concentration signifying the start of senescence [1]. Calmodulin activity has been demonstrated in fruit tissues, and the activator was reported to be inhibited by phenolic compounds naturally occurring in fruit tissues [15]. The present study was undertaken as a first step towards studying the involvement of regulatory calcium-binding proteins in the physiological processes of avocado fruit tissue.

RESULTS AND DISCUSSION

Studies of the significance of calcium during ripening and senescence and in browning disorders of avocado fruit necessitates the isolation of calcium-binding proteins. Avocado proteins with a remarkable range of M_r were found to be retained in a calcium-dependent manner on phenyl sepharose (results not shown). The fact that calcium binding induces hydrophobicity in these proteins might indicate that they exist in a solubilised form in the cytosol under normal physiological conditions, but associated with membranes when increases in the calcium concentration of the cytosol occur. Preliminary results indicated the presence of a prominent, low-M_r, calcium-binding protein; the purification and characterization of which are now reported.

Starting with immature fruit (230–450 g; 20–24 weeks after anthesis), 5.5 mg of the protein (calmodulin) could be isolated from 330 g of acetone powder by ammonium sulphate fractionation at the isoelectric point, hydrophobic interaction chromatography, gel filtration and anion exchange chromatography. The last two purification steps were necessary to remove impurities that co-eluted from the phenyl Sepharose column. Assuming a 50% yield, this implies a content of 11 mg/kg. As avocadoes contain *ca* 0.3% soluble protein on a wet mass basis, it would mean that *ca* 0.3% of the soluble protein is calmodulin, which is in good agreement with accepted results for other tissues [16, 17]. Results with more mature fruit suggest that the calmodulin content decreases as the fruit matures, as only about 30% as much

calmodulin could be isolated from the mature, preclimacteric fruit. This is in accordance with findings of other workers, that calmodulin occurs mostly in actively dividing cells [16–18].

The high lipid and phenolic content of the starting materials made the use of acetone powders essential, and the inclusion of 2-mercaptoethanol during the preparation was necessary to prevent oxidation of phenolic compounds and browning of the extracts. After lyophilization, some difficulty was experienced in dissolving the protein. This was partially alleviated by adding EGTA to the buffer in which it was to be dissolved, or by using high ionic strength buffers.

SDS-PAGE analysis of purified samples of avocado calmodulin in the presence of 1 mM EGTA revealed a single band with a calculated M_r of 18 900 (Fig. 1). Calmodulin retains its calcium-binding properties even in the presence of SDS [19], resulting in the separation of apo- and calcium-saturated forms of calmodulin. This characteristic calcium-dependent shift in electrophoretic mobility was also observed for the protein from avocado.

The calmodulin from avocado fruit, similar to barley calmodulin, shows a doublet on IEF. The pI's for the two bands were calculated as 3.71 and 3.72 respectively, which are in accord with the value of 3.7 reported for calmodulins from *Citrus sinensis*, bovine brain and zucchini [4]. The doublet might be the result of posttranslational modification as it is now known that calmodulin might be subject to modifications such as carboxymethylation, acetylation, the removal of the carboxyl terminal lysine [20], and phosphorylation [21]. Active calmodulins lacking the posttranslational methylation of lysine 115 have also been reported [22]. It is therefore possible that more than one species of calmodulin exist in the fruit tissue of the avocado.

The avocado calmodulin exhibited biological activity as an activator of NAD kinase from pea seedlings. The calmodulins from avocado, wheat germ and bovine brain activated the preparations to approximately the same extent. The concentrations of calmodulin required for half-activation as extrapolated from Fig. 2 is 2.51 μg (avocado), 3.98 μg (wheat germ) and 10 μg (bovine brain).

The activation of cAMP-phosphodiesterase (PDE), (*ca* 28-fold) is regarded as a characteristic property of calmodulins, and therefore this assay was used during the study in an attempt to monitor calmodulin purity and content after various purification steps. In most cases authors found differences of three- to seven-fold in the ability of calmodulins from different sources to activate PDE [3].

Avocado calmodulin was not effective as an activator of the phosphodiesterase from bovine brain; very high concentrations were needed to effect a relatively small activation of *ca* six-fold (Fig. 3). The concentration required for half-activation was 3.7 μM compared to the 7.2 nM of calmodulin from bovine brain; suggesting some important differences in amino acid sequence in the site(s) of interaction with bPDE. The biochemical differences in sensitivity of the NAD kinase and PDE assays have previously been reported [23].

The low activation of bPDE by avocado calmodulin prevented the use of the assay to quantify the calmodulin during the purification. UV spectra and SDS-PAGE were found to be more suitable for identification.

UV spectra of avocado calmodulin were partially obscured by interfering phenolic compounds resulting in a decrease in peak definition. It was difficult to effect complete removal of the compounds, though PVP and

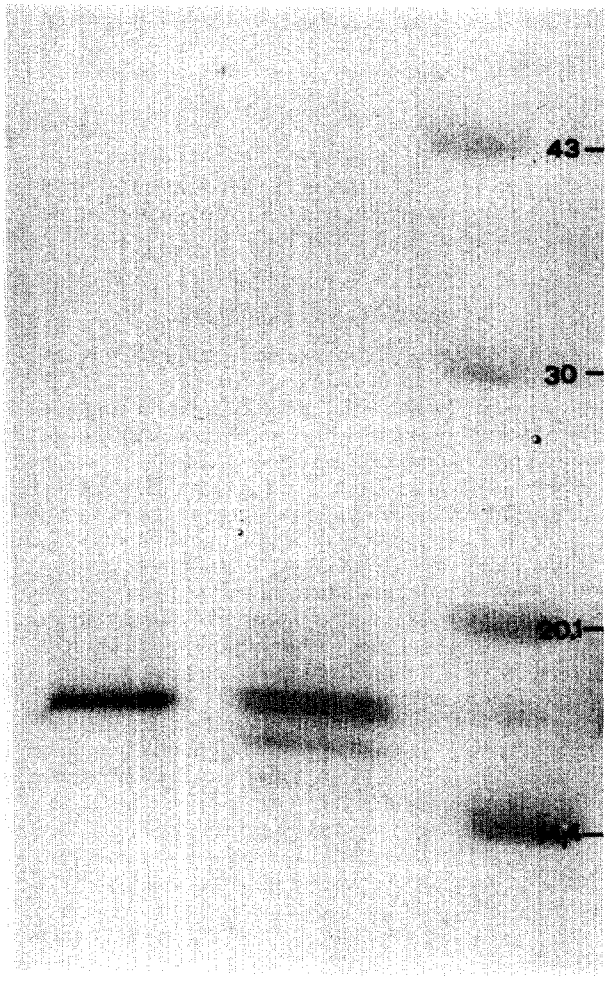

Fig. 1. SDS-PAGE of avocado calmodulin in the presence of 1 mM EGTA (1) and with 0.1 mM $CaCl_2$ in the sample but not in the separating gel (2). Standard proteins (3) with known M_r as indicated.

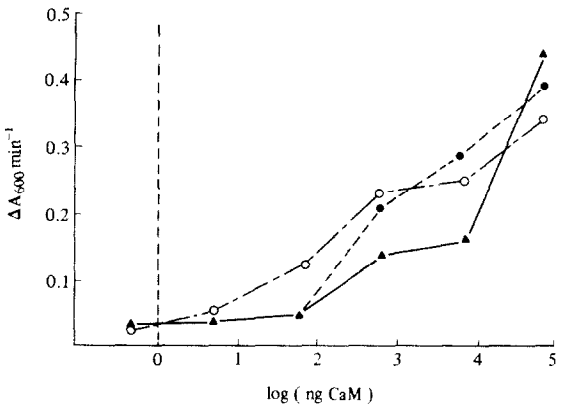

Fig. 2. NAD kinase activation by avocado (- - -), bovine (——) and wheatgerm (·-·-) calmodulin. The assays were performed as described by Matsumoto *et al.* [32].

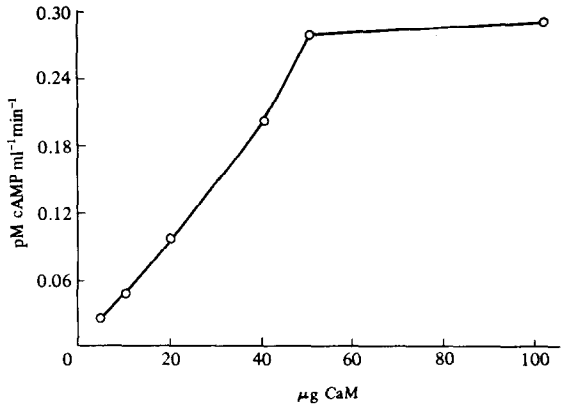

Fig. 3. Activation of cyclic nucleotide monophosphate phosphodiesterase by avocado calmodulin. The reaction conditions are as described in the text.

DEAE chromatography did improve the fine detail in the spectra (Fig. 4). Similar results were obtained for the calmodulin isolated from *Citrus* [4]. The UV spectrum of avocado calmodulin indicates only one tyrosine residue, as was confirmed by amino acid analysis. When excited at 282 nm, a comparatively small (20%) increase in tyrosine fluorescence was observed upon the addition of Ca^{2+} to the protein (not shown), indicating some differences in the primary structure of the tyrosine-containing region [24]. At saturating Ca^{2+}-concentrations, an increase in UV absorbance of *ca* 20% at 276 nm was noted. Mg^{2+} caused less than 10% of the changes in absorbance effected by an equivalent amount of Ca^{2+}. A bathochromic shift of 1.2 nm was noted from 275.2 to 276.4 nm in the presence of Ca^{2+}. This signifies the change to a more hydrophobic conformation, as demonstrated by the higher affinity of the protein in this conformation for phenyl-groups in HIC chromatography. Similar results were reported for bovine brain calmodulin [25].

Circular dicroic spectra were recorded in order to ascertain whether the calcium-dependant conformational changes of the avocado protein were comparable to those of other calmodulins. Separate spectra were obtained for both the far and near UV ranges (Fig. 5a, b). Ca^{2+} was removed by treating the protein with 1 mM EGTA in the desired buffer, and submitting it to buffer exchange using gel filtration on Sephadex G-25. Results were comparable to the findings by other workers [24–26]. Negative peaks at 262 and 269 nm due to phenylalanine [24] were observed in the near UV range. Broad unresolved peaks above 273 nm have been attributed to the tyrosine residue in the calmodulin protein [27]. A change in helical content from an initial 17% in the calcium-depleted conformation to an eventual 24% α-helix in the calcium-saturated form, as calculated from the CD data [28], was noted. The observed differences can possibly be ascribed to this lower helical content of the avocado protein compared to other calmodulins.

The amino acid composition of avocado calmodulin was found to be similar to other higher plant calmodulins such as the proteins of *Spinacea oleracea* [29], *Citrus sinensis* [4] and zucchini [2], and is compared with that of higher and lower animal and plant calmodulins (Table 1). Analysis by OPA-amino acid derivitization indicates a protein of 148 residues while PTH-derivitization suggest a protein of 158 residues. The Asx and Glx residues are slightly higher than the norm for calmodulins. The protein contains 3 mol of histidine and 2 mol of proline per

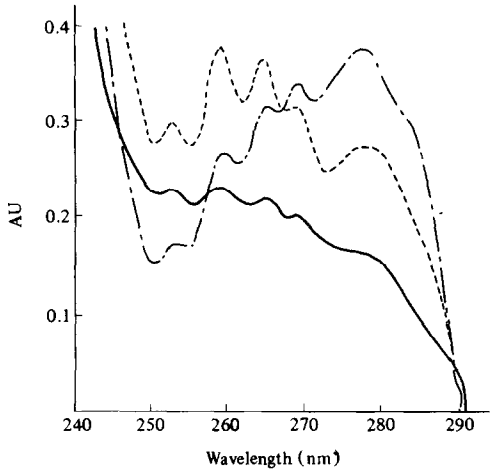

Fig. 4. UV spectra of avocado (——) (0.7 mg/ml), bovine (····) (1 mg/ml) and wheatgerm (- - -) (1 mg/ml) in 50 mM Tris-HCl buffer (pH 7.5).

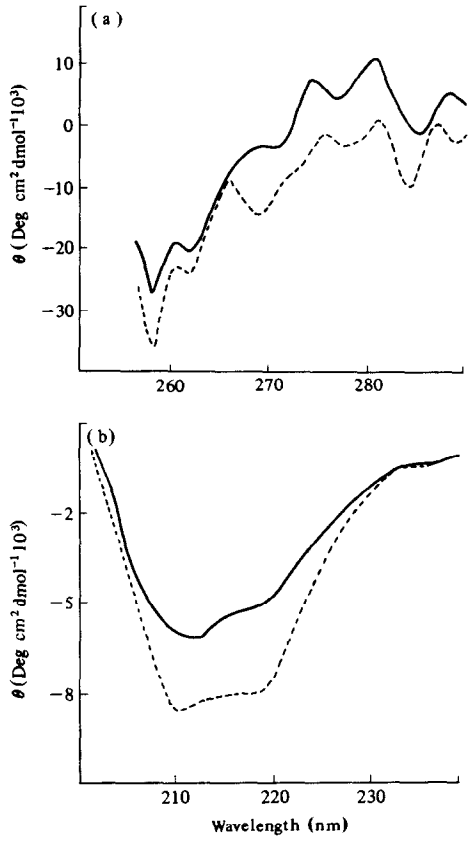

Fig. 5a & b. a) Circular dicroic spectrum of avocado calmodulin (0.7 mg/ml) with 5 mM $CaCl_2$ (····) and without calcium (——) in the near UV range. b) Circular dicroic spectrum of avocado calmodulin (0.7 mg/ml) with 5 mM $CaCl_2$ (····) and without calcium (——) in the far UV range.

Table 1. Comparative amino acid composition of calmodulins [3, 30]

	Avocado (OPA)	Avocado (PTH) derivatives	Bovine brain	Spinach leaves	*Dictiostellium*	*Chlamydomonas*
Asx	24.9	29.7	23	24	24	24
Glx	31.0	29.1	27	27	27	28
Ser	4.4	4.0	4	4	7	5
Gly	12.3	12.0	11	10	11	13
His	3.5	0.6	1	1	1	3
Arg	0.5	5.3	6	5	6	6
Thr	11.1	11.8	12	9	10	12
Ala	12.0	11.2	11	11	10	13
Pro	—	2.2	2	2	2	2
Tyr	1.8	0.9	2	1	2	1
Val	6.4	7.6	7	8	8	8
Met	7.9	6.3	9	8	9	9
Cys	—	0.6	0	1	0	0
Ile	6.8	7.6	8	7	8	6
Leu	9.6	9.0	9	11	10	11
Phe	8.0	7.0	8	9	8	9
Lys	7.2	12.1	7	9	8	12
Lys (Me_3)	1	1	1	1	0	0
Total residues	148	158	148	148	151	162

mol protein. Like the calmodulins from spinach leaves and citrus fruit, the protein contains one mol each of tyrosine, cysteine and trimethyllysine. The ratio of threonine to serine, 3.0, is identical to that of bovine brain but higher than the 2.3 of spinach and the 1.4 of *Dictyostelium*. The protein contains five arginine residues; cleavage at these residues mediated by Arg C endoproteinase [38], resulted in the formation of five peptides. The peptide map (not shown) was essentially identical to that of wheatgerm calmodulin.

We conclude from these results that the protein isolated from avocado fruit mesocarp is a calmodulin that exhibits some apparent differences in biochemical properties compared to well characterized calmodulins from higher plant and animal tissues. It seems to fulfil the same role in calcium-dependent metabolic regulation with similar molecular mechanisms by which the calcium signals are transduced at the cellular level. The role of calmodulin and other calcium-binding proteins in controlling senescence in the ripening fruit needs to be further investigated. Information is especially needed on enzymes involved in ripening which could be regulated *in vivo*, and their respective cellular locations.

EXPERIMENTAL

Materials. Bovine phosphodiesterase (bPDE) was isolated and assayed according to the method of refs [31, 32]. Alkaline phosphatase (AP), adenosine deaminase (ADA) and cAMP were obtained from Boehringer–Mannheim. The concentrations of calmodulin standards from bovine brain were determined spectrophotometrically [33]. Protein was determined by the method of ref. [34] using bovine serum albumin and purified bovine calmodulin as standards.

Purification. Mature preclimacteric avocado fruit (cv. Fuerte) were peeled and diced into crushed ice. Fruit tissue (980 g) was homogenized with 2 vols of Me_2CO containing 0.1% 2-mercaptoethanol and 12 mM HCl at −20°C in a Waring blender. After filtration on a Buchner funnel, the residue was rehomogenized in 300 ml Me_2CO (−20°). Following filtration on a Buchner funnel, the Me_2CO powder was washed (×2) with 200 ml Et_2O at −20°. It was dried *in vacuo* at 0°.

Acetone powder (100 g), was extracted with 20 mM Tris buffer, pH 7.5, containing 0.4 mM EGTA [ethylene glycol-bis-(-aminoethylether) *N*,*N*¹-tetra-acetic acid], 1 mM PMSF(phenylmethyl-sulphonylfluoride) and 15 mM 2-mercaptoethanol. The suspension was homogenized for 1 min, stirred for 30 min at 0°, pressed through a linen cloth and centrifuged for 20 min at 30 000 *g* at 5°. The ppt. was discarded. After adjusting the pH of the resulting supernatant fluid to pH 4 the soln was made to 60% satn with $(NH_4)_2SO_4$ at 4°. The resulting ppt. was collected by centrifugation and suspended in a minimal volume of 50 mM Tris–HCl, pH 7.5 containing 1 mM EGTA, clarified by centrifugation, and the ppt. discarded. The supernatant liquid was heated to 85° for 2 min, then cooled in a icebath. The resulting ppt. was removed by centrifugation, and the supernatant subjected to calcium-induced hydrophobic interaction chromatography on phenyl Sepharose at room temp., essentially as described in ref. [35]. Calmodulin was desorbed from the column by the inclusion of EGTA in the buffer.

The calmodulin containing fractions from the phenyl Sepharose step were combined and lyophilized. The lyophilized powder was dissolved in a minimum of 50 mM NH_4OAc buffer, pH 6.0, and subjected to gel exclusion chromatography on Sephadex G-75 F (40.0 × 2.5 cm column) at 4° and a flow rate of 15 cm/hr (75 ml/hr).

Calmodulin was isolated from the second peak obtained in the gel filtration step by chromatographing on a column of DEAE-cellulose (16 mm × 200 mm) according to the method of ref. [36]. Calmodulin eluted at about 0.3 M NaCl.

Electrophoresis and isoelectric focussing. SDS-PAGE was performed according to the method of ref. [37] on 12.5% gels.

Samples contained 1 mM EGTA or 0.1 mM $CaCl_2$, but no calcium was included in the separating gels due to problems caused by precipitation with SDS. Gels were stained with 0.25% Coomassie Blue G-250. From 40–300 μg (20–50 μl) protein was applied per lane. Run time was 4.5 hr (Fig. 1).

IEF was performed using a LKB Flatbed electrophoresis unit and 5% gels in the pH interval 2.5–5 were prepared. Gels were prefocussed for 500 Vhr at 6 W. Focusing was performed for 3000 Vhr at 30 W, with a interelectrode distance of 8 cm. pI Marker kits from Pharmacia were used to determine pI's of the focussed proteins. Staining was with Coomassie Blue R-250.

Amino acid analysis. Amino acid composition was determined with a Waters HPLC Amino Acid Analyzer using both the *o*-phtalaldehyde (OPA) and phenylthiohydantoin (PTH) systems. Samples of 100 μg of calmodulin were subjected to gas phase hydrolysis after addition of a crystal of PhOH. Sample preparation was done according to the procedure detailed in the PICO-TAG (Waters) manual. Duplicate samples containing the equivalent of 10 μg (0.55 nmol) of calmodulin was injected after derivatization.

Protease digestion and peptide mapping. Cleavage at arginine residues was mediated by Arg C endoproteinase (Boehinger–Mannheim) according to the instructions of the manufacturer. Separation of the resulting peptides was performed with a reverse phase column (Whatman ODS-2 10 mm × 250 mm) as described in ref. [38].

UV and circular dichroism spectra. The UV spectra of calmodulin from bovine brain, wheat germ and avocado mesocarp were obtained. Spectra of calmodulin were recorded in the presence and absence of calcium (5 mM).

Circular dichroism spectra: a minimum of two replicates were done for each sample, and the value for the corresponding blank subtracted before plotting the spectra.

Phosphodiesterase assay. Phosphodiesterase was prepared from bovine brain as described in ref. [32]. PDE was obtained by eluting the column with 50 mM NaOAc, pH 5.5. The enzyme was activated 24 to 32-fold by calmodulin from beef brain. The assay was performed according to the method of ref. [31]. The decrease in A_{265} was monitored, and activity determined as defined in ref. [33].

NAD kinase activation. NAD kinase was isolated and partially purified according to the method described in ref. [16] from pea seedlings. The method of ref. [32] was used for assaying the activity. The assay was standardized using calmodulin purified from bovine brain and wheat germ.

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